RESEARCH ARTICLE

# The *Saccharomyces* SUN gene, *UTH1*, is involved in cell wall biogenesis

James J. Ritch, Shawn M. Davidson, Jared J. Sheehan & Nicanor Austriaco, O.P.

Department of Biology, Providence College, Providence, RI, USA

**Correspondence:** Nicanor Austriaco, O.P. Department of Biology, Providence College, 1 Cunningham Sq., Providence, RI 02918, USA. Tel.: +1 401 865 1823; fax: +1 401 865 2959; e-mail: naustria@providence.edu

**Present addresses:** James J. Ritch, MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital, 114 16th St., Charlestown, MA 02129, USA. Jared J. Sheehan, Cancer Immunology & AIDS, Dana-Farber Cancer Institute, 44 Binney St., JFB 703, Boston, MA 02115, USA.

Received 9 August 2009; revised 6 December 2009; accepted 13 December 2009.
Final version published online 12 January 2010.

DOI:10.1111/j.1567-1364.2009.00601.x

Editor: Ian Dawes

**Keywords**
*UTH1*; *PKC1*; SUN genes; cell wall biogenesis; β-D-glucan; chitin.

## Abstract

Deletion of the *Saccharomyces* gene, *UTH1*, a founding member of the *SUN* family of fungal genes, has pleiotropic effects. Several phenotypes of Δ*uth1* cells including their decreased levels of mitochondrial proteins, their impaired autophagic degradation of mitochondria, and their increased viability in the presence of mammalian BAX, a proapoptotic regulator localized to the mitochondria, have prompted others to propose that the Uth1p functions primarily at the mitochondria. In this report, we show that cells lacking *UTH1* have more robust cell walls with higher levels of β-D-glucan that allows them to grow in the presence of calcofluor white or sodium dodecyl sulfate, two reagents known to perturb the yeast cell wall. Moreover, these Δ*uth1* cells are also significantly more resistant to spheroplast formation induced by zymolyase treatment than their wild-type counterparts. Surprisingly, our data suggest that several of the enhanced growth phenotypes of Δ*uth1* cells, including their resistance to BAX-mediated toxicity, arise from a strengthened cell wall. Therefore, we propose that Uth1p's role at the cell wall and not at the mitochondria may better explain many of its effects on yeast physiology and programmed cell death.

## Introduction

A founding member of the SUN (*SIM1*, *UTH1*, and *NCA3*) family of fungal genes, *UTH1* was originally identified in a genetic screen for *Saccharomyces cerevisiae* mutants that increased the stress resistance and the replicative lifespan of yeast cells (Kennedy *et al.*, 1995; Austriaco, 1996). Mutant cells lacking *UTH1* have longer replicative lifespans (Kennedy *et al.*, 1995; Austriaco, 1996); are capable of growing at elevated temperatures (Austriaco, 1996; Camougrand *et al.*, 2003); are resistant to hydrogen peroxide (Austriaco, 1996; Bandara *et al.*, 1998) and rapamycin (Camougrand *et al.*, 2003); and are sensitive to copper (Jo *et al.*, 2008) and to paraquat (Austriaco, 1996; Bandara *et al.*, 1998). Finally, Δ*uth1* cells are also able to grow in the presence of overexpressed proapoptotic mammalian BAX, suggesting that *UTH1* may be involved in the regulation of yeast-programmed cell death (Camougrand *et al.*, 2003).

What does Uth1p do? Several lines of evidence have prompted Camougrand and colleagues to propose that the Uth1p protein functions primarily at the mitochondria. First, they showed that the inactivation of *UTH1* reduces the levels of mitochondrial proteins including cytochrome $aa_3$, *c*, and *b*, and citrate synthase (Camougrand *et al.*, 2000). Next, they localized Uth1p to the outer mitochondrial membrane and to the cell wall (Velours *et al.*, 2002). Third, as we have already noted above, they observed that deleting *UTH1* allows yeast cells to survive the overexpression of mammalian BAX, a proapoptotic protein known to act at yeast mitochondria (Camougrand *et al.*, 2003). Finally, they discovered that the autophagic degradation of mitochondria is impaired in Δ*uth1* cells (Kissova *et al.*, 2004, 2007). In light of these data, Camougrand *et al.* (2004) have suggested that Uth1p is a regulator of mitochondrial function and that this role may explain its diverse effects on yeast apoptosis and cell physiology.

In this paper, we report that *UTH1* is involved in cell wall biogenesis: cells lacking *UTH1* have more robust cell walls that are relatively more resistant to enzymatic attack by zymolyase than their wild-type counterparts, probably

because they contain higher levels of β-D-glucan. Surprisingly, our data also suggest that several of the enhanced growth phenotypes of Δ*uth1* cells, including their resistance to BAX-mediated toxicity, arise from a strengthened cell wall. Therefore, we propose that Uth1p's role at the cell wall and not at the mitochondria may better explain many of its effects on yeast physiology and programmed cell death.

## Materials and methods

### Yeast strains, plasmids, and growth conditions

All experiments were performed with isogenic strains in the W303-1A background (MATa *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *ssd1-d2*). The Δ*uth1* mutant was created by disrupting the ORF with the kanR marker using a PCR-based knockout strategy (Brachmann *et al.*, 1998) and verified both by PCR and by phenotypic analysis. To overexpress either human BAX or yeast *PKC1* in our strains, we transformed either plasmid pCM189/Bax (Camougrand *et al.*, 2003) or plasmid pFR22 (Roelants *et al.*, 2004) into our cells and plated them on selective media. Doxycycline supplementation was used to regulate BAX expression as described previously (Camougrand *et al.*, 2003). For all the experiments described in this paper, cells were cultured using standard protocols (Amberg *et al.*, 2005), and transformations were accomplished using the lithium acetate method (Gietz & Schiestl, 2007). Unless noted otherwise, all drugs were purchased from Sigma-Aldrich.

### Spot assays

Cells were grown overnight in either rich or selective media at 30 °C and then diluted to a final concentration (an approximate $OD_{660\,nm}$ value of 0.2). For each strain, a series of 10-fold dilutions was then prepared in water over a range of concentrations from $10^{-1}$ to $10^{-5}$ relative to the initial culture. Spots of 5 μL from each dilution series were then plated on the indicated media and cultured at either 30 or 39 °C for 2, 3, or 5 days, depending on the particular plate. Plates supplemented with drugs were poured and used on the same day. All spot assays were repeated at least three times and a representative experiment is shown.

### Spheroplast rate assay

Enzyme preparation and cell wall lysis assay were based on the method described previously (Ovalle *et al.*, 1998). Briefly, cells were grown overnight in rich media at 30 °C, harvested, and washed three times with deionized water, and resuspended to an $OD_{660\,nm}$ of 0.5 in TE buffer, pH 7.5 (50 mM Tris/HCl, 5 mM EDTA). Zymolyase (5 U $\mu L^{-1}$; Zymo Research, Orange, CA) was then added to the cells to a final concentration of 12 μg $mL^{-1}$. Cell suspensions were incubated at 23 °C and their OD was recorded at 4-min intervals for the indicated time period.

### Analysis of cell wall sugar composition

Chitin levels were quantified according to a method described previously (Lesage *et al.*, 2005). Yeast cultures were first grown to the stationary phase in liquid yeast peptone dextrose (YPD) medium and then diluted 1 : 100 in fresh YPD and incubated at 30 °C with shaking for 18–22 h. Typically, 1 mL of culture was spun in a microfuge tube at 14 K for 2 min and the media were removed. The cell pellets were then air dried at 37 °C for 2–3 days. Next, cell pellets were resuspended in 1 mL of 6% KOH and heated at 80 °C for 90 min with occasional mixing. Alkaline-insoluble material was pelleted (20 000 ***g***, 20 min) and neutralized with phosphate-buffered saline for 10–20 min with occasional mixing. After centrifugation (20 000 ***g***, 20 min), 200 μL of McIlvaine's Buffer (0.2 M $Na_2HPO_4$/0.1 M citric acid, pH 6.0) was added to the pellets. Extracts were then stored at − 20 °C until processed for chitin measurements. Samples were thawed and subjected to digestion with 5 μL of 5 mg $mL^{-1}$ chitinase from *Trichoderma viride* (Sigma-Aldrich) at 37 °C for 36–40 h and then for 20–24 h. The amounts of chitin were then determined using a modified Morgan–Elson procedure as described previously (Bulik *et al.*, 2003). The levels of chitin, expressed as GlcNAc concentrations, were then normalized to the dry weight of the sample.

Next, β-D-glucan levels were quantified according to the methods of Boone *et al.* (1990) and Yiannikouris *et al.* (2004). Yeast cells were grown as 10-mL cultures of YPD until the stationary phase. Cells were harvested, washed once with distilled water, and then extracted three times with 0.5 mL of 3% NaOH at 75 °C (1 h per extraction). After alkali extraction, the alkali-insoluble material was washed once with 1 mL of 100 mM Tris-HCl (pH 7.5) and once with 1 mL of 10 mM Tris-HCl (pH 7.5). The washed residue was then digested for 16 h at 37 °C with 1 mg of Zymolyase 100T (United States Biological, Swampscott, MA) in 1 mL of 10 mM Tris-HCl (pH 7.5). The insoluble pellet that remains after zymolyase digestion was removed by centrifugation, and the supernatant was dialyzed against distilled water, using Spectra/por tubing with a 6000–8000-D pore size (Spectrum Laboratories, Rancho Dominguez, CA), for 16 h. The carbohydrate content before dialysis [(1,3) plus (1,6) β-glucan] and post dialysis [(1,6) β-glucan alone] was measured as hexose using the phenol–sulfuric acid method (Dubois *et al.*, 1956). The levels of β-D-glucan were then normalized to the dry weight of the sample. Finally, supernatants obtained from the alkali extraction containing alkali soluble β-glucans and mannoproteins from yeast cell walls were dialyzed (1 : 100, v/v) with 0.02 M Tris-HCl buffer (pH

7.4) for at least 16 h at 4 °C with 0.02 M Tris-HCl buffer (pH 7.4). Mannans and β-glucans were separated on a concanavalin A Sepharose column (Pharmacia) at 4 °C as described previously (Yiannikouris *et al.*, 2004). Alkali-soluble β-glucans were eluted with 0.02 M Tris-HCl buffer (pH 7.4)/0.5 M NaCl and stored at −20 °C until the carbohydrate content was measured as above, using the phenol–sulfuric acid method.

## Results and discussion

### Deleting *UTH1* has pleiotropic effects including phenotypes associated with the endoplasmic reticulum (ER)

Deletion of the gene, *UTH1*, has pleiotropic effects. As we and others have shown previously, mutant cells lacking *UTH1* are capable of growing at elevated temperatures; are resistant to hydrogen peroxide; and are sensitive to copper and to paraquat (Fig. 1). In addition, we have also discovered that they are resistant to β-mercaptoethanol (Fig. 1) and to dithiothreitol (data not shown), drugs known to induce ER stress (Cox & Walter, 1996). All of these phenotypes suggest that *UTH1* function involves numerous physiological processes in the cell.

### *UTH1* is involved in maintaining the integrity of the yeast cell wall

*UTH1* is a founding member of the SUN family of fungal genes (Austriaco, 1996). Four other fungal SUN genes, *SUN4* in *S. cerevisiae* (Mouassite *et al.*, 2000), *psu1* in *Saccharomyces pombe* (Omi *et al.*, 2005), and *SUN41* and *SIM1*/*SUN42* in *Candida albicans* (Firon *et al.*, 2007; Hiller *et al.*, 2007; Sosinska *et al.*, 2008), have been implicated in the regulation of the integrity of the yeast cell wall. To test whether *UTH1* has a similar function, we plated wild-type and Δ*uth1* cells on media containing either calcofluor white (CFW) or sodium dodecyl sulfate (SDS), two reagents known to perturb the yeast cell wall (Kaeberlein & Guarente, 2002), and observed that the mutant is more resistant to these agents than the wild-type strain (Fig. 2a). This suggested that deleting *UTH1* strengthens the yeast cell wall.

To further characterize this phenotype, we plated wild-type and Δ*uth1* cells on CFW and on SDS plates supplemented with 1 M sorbitol, which provides osmotic stabilization and prevents lysis caused by a weakened cell wall (Kaeberlein & Guarente, 2002). Under these conditions, there is no difference in the growth of our wild-type and mutant strains, suggesting that the deletion of *UTH1* phenocopies the supplementation of growth media with an osmotic stabilizer (Fig. 2a).

To confirm our findings, we overexpressed *PKC1* in wild-type and Δ*uth1* cells using a high-copy plasmid and plated

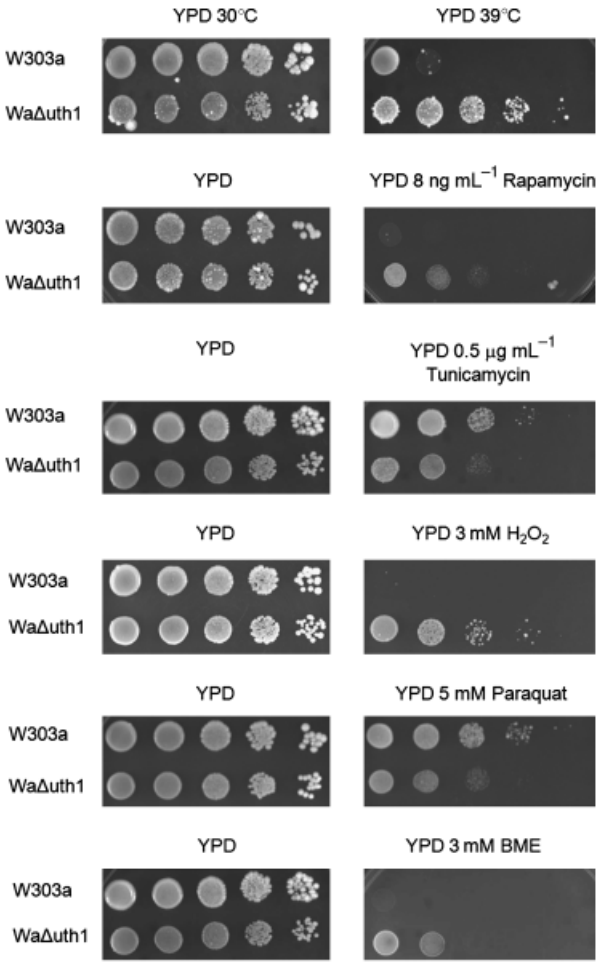


**Fig. 1.** *UTH1* is involved in the yeast cell's response to different stresses. Aliquots (5 μL) of 10-fold serial dilutions of wild-type and Δ*uth1* mutant cells in the W303-1A strain background were plated onto the designated media and cultured at either 30 or 39 °C for 2, 3, or 5 days, depending on the particular plate. All spot assays were repeated at least three times, and a representative experiment is shown.

them on SDS plates with or without osmotic stabilization. Pkc1p is a central integrator of cell integrity that acts to promote transcription of cell wall biosynthetic genes (Heinisch *et al.*, 1999). We observed that the growth rates of wild-type cells, wild-type cells overexpressing *PKC1*, and Δ*uth1* cells are indistinguishable on SDS plates supplemented with sorbitol, suggesting that deleting *UTH1* and overexpressing *PKC1* have the same effect of enhancing cell wall integrity (Fig. 2b). Because we see no difference in growth between wild-type cells overexpressing *PKC1*, wild-type cells on sorbitol, and wild-type cells overexpressing *PKC1* on sorbitol, on SDS plates, our data suggest that the phenotypes we observe from either of these manipulations – either the overexpression of *PKC1* or the supplementation of media with 1 M sorbitol – can be attributed directly to their enhancement of cell wall integrity rather than to an indirect effect caused either by a

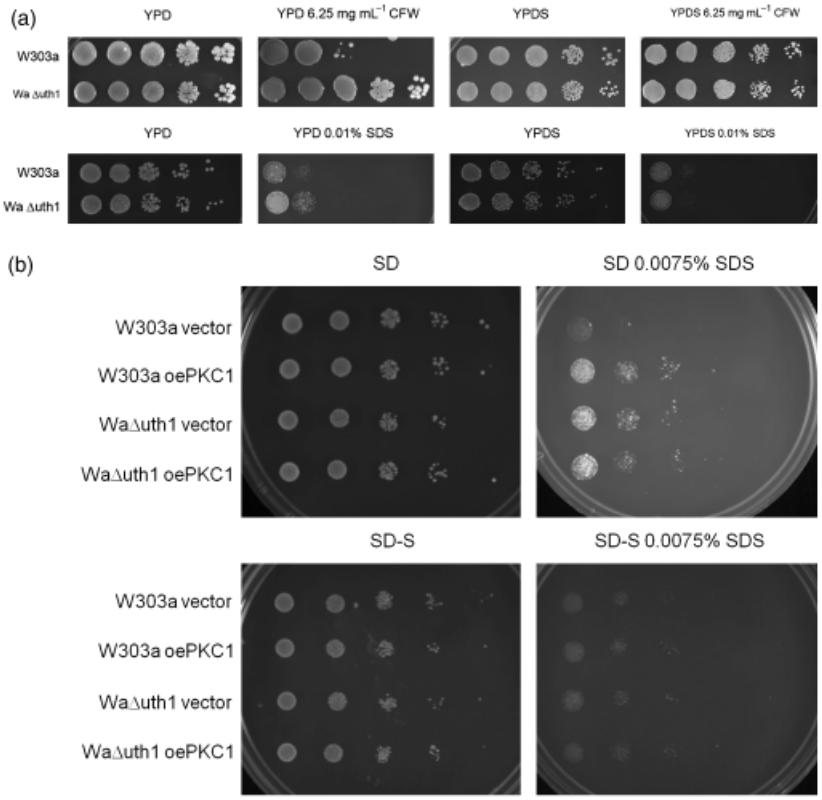


**Fig. 2.** *UTH1* is involved in maintaining the integrity of the yeast cell wall. (a) Deleting *UTH1* enhances cell growth on media supplemented with CFW or SDS. Aliquots (5 μL) of 10-fold serial dilutions of wild-type and Δ*uth1* mutant cells in the W303-1A strain background were plated onto the designated media and cultured at 30 °C for 2 or 4 days, depending on the particular plate. YPDS denotes YPD plates supplemented with 1 M sorbitol, respectively. (b) Overexpression of *PKC1* enhances cell wall integrity, mimicking a deletion of *UTH1*. Aliquots (5 μL) of 10-fold serial dilutions of wild-type and Δ*uth1* mutant cells transformed with plasmid pFR22 to overexpress *PKC1* were plated onto the designated selective synthetic dextrose (SD) plates and cultured at 30 °C for 2 or 5 days. SD-S denotes SD plates supplemented with 1 M sorbitol. All spot assays were repeated at least three times, and a representative experiment is shown.

non-cell-wall-related function of overexpressed *PKC1* (Fairn *et al.*, 2007) or by the sorbitol-mediated activation of the osmoregulatory high osmolarity glycerol pathway (Saito & Tatebayashi, 2004). Otherwise, we would have observed a synergistic effect on the growth of wild-type cells overexpressing *PKC1* on SDS plates supplemented with sorbitol.

Finally, to more directly assay the structural integrity of the wild-type and Δ*uth1* yeast cell walls, we compared the rates of formation of spheroplasts of wild-type and mutant Δ*uth1* yeast cells cultured in a hypotonic solution in the presence of zymolyase, a mixture of cell wall-digesting enzymes (Ovalle *et al.*, 1998). This spheroplast rate assay has been used by others to show that Acb1p (Gaigg *et al.*, 2001), Pho85p (Huang *et al.*, 2002), and Bet1p (Kipnis *et al.*, 2004) are all involved in maintaining the integrity of the yeast cell wall. As shown in Fig. 3, mutant cells lacking *UTH1* were significantly more resistant to zymolyase treatment than wild-type cells. More specifically, for wild-type cells, the mean lag time (LT) in the presence of zymolyase for three independent samples – where LT has been estimated by interpolation of the lysis curve as the time in which the $OD_{660\,nm}$ declined by 0.05 from its initial value (Ovalle *et al.*, 1998) – was 12 min and the mean maximal lysis rate (MLR) was 0.230 – where MLR has been defined as the absolute value of the slope of the least-squares fit line for the portion of the lysis curve with the steepest log-linear decline (Ovalle *et al.*, 1998). In contrast,

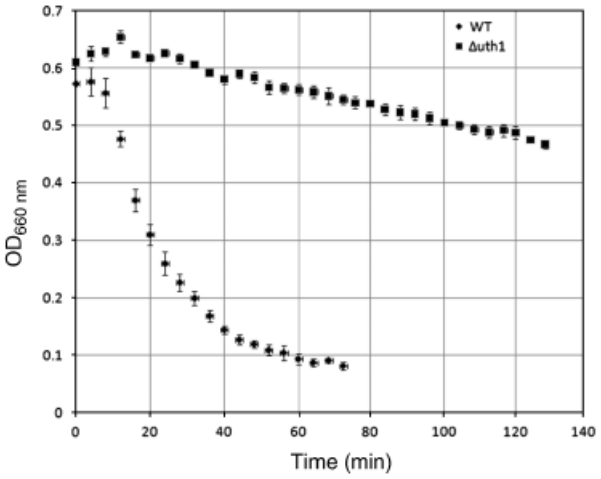


**Fig. 3.** Deleting *UTH1* decreases the rate of spheroplast formation of yeast cells. Cells of the indicated genotypes were cultured overnight in rich YPD media at 30 °C, harvested, and washed three times with deionized water, and then resuspended to an $OD_{660\,nm}$ of 0.5 in TE buffer, pH 7.5 (50 mM Tris/HCl, 5 mM EDTA). Zymolyase (5 U μL$^{-1}$; Zymo Research) was then added to the cells to a final concentration of 12 μg mL$^{-1}$. Cell suspensions were incubated at 23 °C and their $OD_{660\,nm}$ was recorded at 4-min intervals for the indicated time period. Assays were performed in triplicate, and the mean values are shown along with the standard deviation (SD).

for the Δ*uth1* mutants, the mean LT for three independent samples was 18.6 min and the MLR was 0.074. The rate indices (RI) of the wild-type and Δ*uth1* cells, where RI has been defined as MLR/LT (Ovalle *et al.*, 1998), were 0.0192 and 0.0041, respectively ($P < 0.002$). These results demonstrate that the walls of Δ*uth1* cells are more resistant to enzymatic attack than those of wild-type controls, confirming the findings from the genetic analysis that had suggested that the cell walls of Δ*uth1* cells are more robust.

Taken together, both our genetic and biochemical data suggest that *UTH1*'s function parallels the role of four other fungal SUN genes: *psu1*, which is involved in cell wall synthesis in *S. pombe* (Omi *et al.*, 2005); *SUN4*, which is involved in cell septation and that, significantly, appears to act in concert with *UTH1* (Mouassite *et al.*, 2000); and *SUN41* and *SIM1*/*SUN42*, which have been implicated in cell wall remodeling in *C. albicans* (Firon *et al.*, 2007). In some way, *UTH1* is involved in the biogenesis of the cell wall in *S. cerevisiae*.

## Deleting *UTH1* alters the polysaccharide composition of the yeast cell wall

How does *UTH1* regulate the integrity of the yeast cell wall? Firon *et al.* (2007) have shown that *UTH1* is able to complement a *sun41*ΔΔ *sun42*Δ double mutant in *C. albicans*. Given that *SUN41* and *SIM1*/*SUN42* mutants manifest specific cell wall defects at the septa in *Candida* and that these *Candida* mutants are sensitive only to cell wall-perturbing agents that are specific to chitin synthesis, these authors have proposed that *UTH1* is involved in chitin biosynthesis in *S. cerevisiae*. To test this hypothesis, we determined the chitin levels in the cell walls of Δ*uth1* cells and showed that they indeed have decreased amounts of chitin as compared with wild-type controls (Table 1).

This finding – that Δ*uth1* cells have lower chitin levels – was unexpected. As shown in Figs 2 and 3, *Saccharomyces* cells lacking *UTH1* are not only more resistant to CFW and to SDS, two reagents that are known to destabilize the cell wall (Kaeberlein & Guarente, 2002), but are also significantly more resistant to zymolyase, a mixture of cell wall-digesting enzymes (Kitamura & Yamamoto, 1972). This would *not* be expected if deleting *UTH1* only reduces the chitin content of the cell wall, an effect expected to weaken and not to strengthen the cell wall. Interestingly, one previous report has shown that mutant cells with increased resistance to CFW have lower chitin levels than their wild-type counterparts (Roncero *et al.*, 1988).

How could lower levels of chitin lead to the strengthening of the yeast cell wall? Some have suggested that a compensatory mechanism exists in yeast in response to cell wall damage, whereby decreases in β-D-glucan levels lead to a compensatory increase in chitin levels (Kapteyn *et al.*, 1997; Popolo *et al.*, 1997; Ram *et al.*, 1998; Valdivieso *et al.*, 2000). To explain our data, therefore, we predicted that the reverse mechanism may also exist: the lower levels of chitin in Δ*uth1* cells may be accompanied by compensatory higher levels of β-D-glucan. To test this hypothesis, we determined the β-D-glucan levels of Δ*uth1* cells. Indeed, as predicted, these mutant Δ*uth1* cells had cell walls with significantly higher levels both of total alkaline-soluble and total alkaline-insoluble β-D-glucan and of alkaline-insoluble (1,6) β-D-glucan more specifically (Table 1). This would explain why Δ*uth1* cells are more resistant to zymolyase, a mixture of cell wall-digesting enzymes composed primarily of β-1,3-glucan laminaripentaohydrolase and β-1,3-glucanase (Kitamura & Yamamoto, 1972). The higher levels of β-D-glucan would also explain our genetic data that had suggested that the cell walls of Δ*uth1* cells are more robust than their wild-type counterparts.

In sum, our data suggest that *UTH1* is involved in the biogenesis of the yeast cell wall. The precise molecular mechanism behind this role, however, remains unknown: Uth1p could be involved in any of the regulatory pathways that have been linked to the complex process of cell wall assembly in *S. cerevisiae* (Lesage & Bussey, 2006). It is significant that the Uth1p homolog in *C. albicans*, Sim1p, has been identified as a covalently linked cell wall protein by MS (Sosinska *et al.*, 2008). Another group has also shown that Uth1p appears to act in concert with another SUN protein, Sun4p, to contribute to cell

**Table 1.** Chitin and glucan composition of the cell wall

| | | Glucan | | |
|---|---|---|---|---|
| | | Alkaline insoluble | | Alkaline soluble |
| | Chitin<br>Total chitin (nmol GlcNAc $mg^{-1}$ dry weight) | Total β-glucan (μg $g^{-1}$ dry weight) | (1,6) β-glucan (μg $g^{-1}$ dry weight) | Total β-glucan (μg $g^{-1}$ dry weight) |
| W303-1A | 21.3 (1.75) | 190 (28) | 51 (8) | 21 (44) |
| Δ*uth1* | 15.9 (1.34)<br>($P < 0.002$) | 488 (52)<br>($P < 0.001$) | 90 (22)<br>($P < 0.05$) | 247 (109)<br>($P < 0.05$) |

Deleting *UTH1* alters the polysaccharide content of the yeast cell wall. Chitin and β-D-glucan levels were quantified as described in Materials and methods. Assays were performed in triplicate, and the mean values are shown along with the standard deviation (SD) in parentheses. Statistical significance was calculated using Student's unpaired *t*-test.

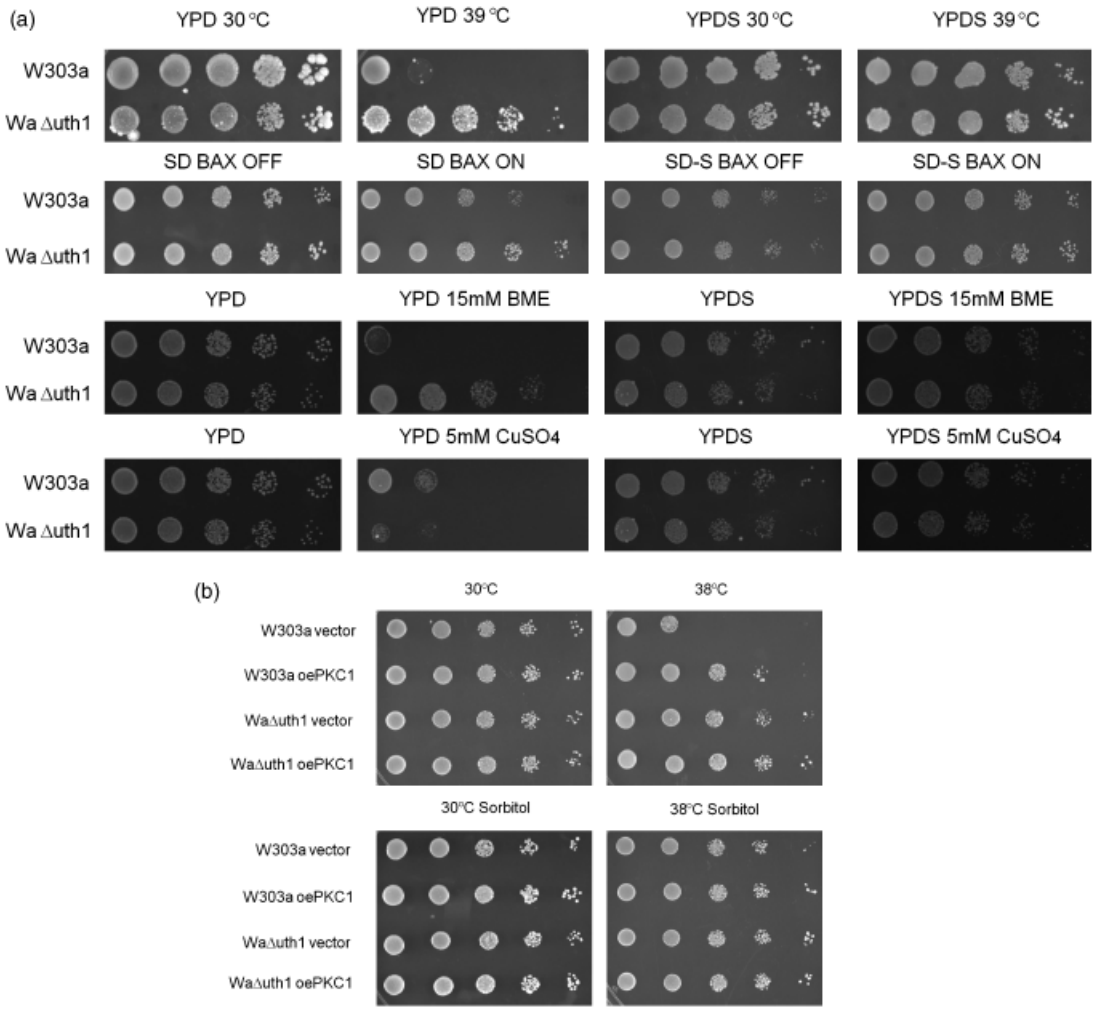


**Fig. 4.** Deleting *UTH1* enhances the cell's response to diverse stresses by strengthening its cell wall. (a) Osmotic stabilization with 1 M sorbitol phenocopies a deletion of *UTH1*. Aliquots (5 μL) of 10-fold serial dilutions of wild-type and Δ*uth1* mutant cells were plated onto the designated media and cultured at either 30 or 39 °C for 2, 3, or 5 days. YPDS and SD-S denote YPD and SD plates supplemented with 1 M sorbitol, respectively. To overexpress human BAX in our strains, we transformed plasmid pCM189/Bax into our cells and plated them on selective SD media with or without doxycycline supplementation as described previously (Camougrand *et al*., 2003). (b) Overexpression of *PKC1* enhances cell growth at elevated temperatures, mimicking a deletion of *UTH1*. Aliquots (5 μL) of 10-fold serial dilutions of wild-type and Δ*uth1* mutant cells transformed with plasmid pFR22 to overexpress *PKC1* were plated onto the designated selective SD plates and cultured at 30 or 38 °C for 2 or 5 days. All spot assays were repeated at least three times, and a representative experiment is shown.

wall morphogenesis and septation, strongly supporting our proposal that the protein is involved in cell wall biogenesis (Mouassite *et al*., 2000). Finally, it is striking that two research teams have shown that *UTH1* belongs to a set of Ssd1p-associated mRNAs that is enriched in cell wall related genes (Hogan *et al*., 2008; Jansen *et al*., 2009).

## Deleting *UTH1* improves growth under diverse stress conditions by enhancing cell wall integrity

Finally, to determine whether *UTH1*'s role in regulating the integrity of the yeast cell wall could explain any of the pleiotrophic phenotypes of the Δ*uth1* mutant, we took wild-type and Δ*uth1* cells and stressed them on media with or without sorbitol supplementation. We discovered that there is no difference in growth between these two strains either at 39 °C, on media containing either β-mercaptoethanol or copper, or with overexpressed mammalian BAX, as long as they are cultured on media with an osmotic stabilizer (Fig. 4a). This suggested that the growth enhancement attributed to the deletion of *UTH1* under these conditions could be explained by the gene's role in cell wall biogenesis rather than on mitochondrial function. This was true regardless of whether the BAX cell death assays were

performed on sorbitol-supplemented media containing either glucose or glycerol as a carbon source (data not shown). In both cases, the viability of wild-type and Δ*uth1* cells overexpressing mammalian BAX was indistinguishable.

To confirm this result, we repeated the assay with wild-type and Δ*uth1* cells, both overexpressing *PKC1* on a high-copy plasmid, and observed that the growth rates of wild-type cells, wild-type cells overexpressing *PKC1*, and Δ*uth1* cells, at 38 °C, are indistinguishable on plates supplemented with sorbitol, suggesting once again that the enhanced growth phenotype of Δ*uth1* cells at elevated temperatures is linked to the enhancement of cell wall integrity (Fig. 4b). As before, the absence of a synergistic effect on the growth at 38 °C of wild-type cells overexpressing *PKC1* on plates supplemented with sorbitol suggested that the growth enhancement phenotype could be attributed directly to a strengthened cell wall. Finally, parallel results were obtained on copper-supplemented plates, suggesting that the sensitivity of Δ*uth1* cells to copper is also mediated by the gene's effect on the integrity of the cell wall (data not shown). It would be interesting to determine whether any of the other phenotypes of Δ*uth1* cells, especially those associated with Uth1p's putative mitochondrial function, can also be linked to its role in regulating the integrity of the yeast cell wall. In light of our findings, it is intriguing to note that two groups have been unable to find the reported link between Uth1p function and mitochondrial autophagy (Kanki *et al.*, 2009; Okamoto *et al.*, 2009). However, it is still not clear whether this was due to differences in the strain background. If so, we speculate that Uth1p's differential effects on mitophagy could be linked to the differences in cell wall composition commonly seen among wild-type yeast strains.

In conclusion, we show that cells lacking *UTH1* have more robust cell walls that are resistant to zymolyase treatment because they contain higher levels of β-D-glucan. Surprisingly, our data also suggest that several of the enhanced growth phenotypes of Δ*uth1* cells, including their resistance to BAX-mediated toxicity, arise from a strengthened cell wall. Thus, we propose that Uth1p's role at the cell wall and not at the mitochondria may better explain many of its effects on yeast physiology and programmed cell death.

## Acknowledgements

We thank Mary Blasik, Gina Contuzzi, Kathleen Cornely, David J. Laprade, Kevin R. Murphy, and Charles Specht (University of Massachusetts School of Medicine, Worchester, MA) for technical assistance, and Brian K. Kennedy (University of Washington, Seattle), Stephen Manon (University of Bordeaux), and Jeremy Thorner (University of California, Berkeley) for strains and/or plasmids. We also thank both Gregory Liszt and Leonard Guarente (MIT) and Charles Specht (University of Massachusetts School of Medicine, Worchester, MA) for helpful discussion. Our laboratory is supported by Rhode Island INBRE Grant P20RR016457 from the National Center for Research Resources (NCRR) of the National Institutes of Health (NIH) and a CAFR grant from Providence College.

## Authors' contribution

J.J.R. and S.M.D. contributed equally to this work. J.J.R., S.M.D., and N.A. performed the experiments. J.J.S. constructed yeast strains. J.J.R., S.M.D., and N.A. designed experiments, analyzed data, and wrote the manuscript.

## References

Amberg DC, Burke D & Strathern JN (2005) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Austriaco N (1996) *UTH1* and the genetic control of aging in the yeast, *Saccharomyces*. PhD Dissertation, Massachusetts Institute of Technology, MA.

Bandara PD, Flattery-O'brien JA, Grant CM & Dawes IW (1998) Involvement of the *Saccharomyces cerevisiae* UTH1 gene in the oxidative-stress response. *Curr Genet* **34**: 259–268.

Boone C, Sommer SS, Hensel A & Bussey H (1990) Yeast KRE genes provide evidence for a pathway of cell wall beta-glucan assembly. *J Cell Biol* **110**: 1833–1843.

Brachmann CB, Davies A, Cost GJ *et al.* (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**: 115–132.

Bulik DA, Olczak M, Lucero HA, Osmond BC, Robbins PW & Specht CA (2003) Chitin synthesis in *Saccharomyces cerevisiae* in response to supplementation of growth medium with glucosamine and cell wall stress. *Eukaryot Cell* **2**: 886–900.

Camougrand N, Grelaud-Coq A, Marza E, Priault M, Bessoule JJ & Manon S (2003) The product of the UTH1 gene, required for Bax-induced cell death in yeast, is involved in the response to rapamycin. *Mol Microbiol* **47**: 495–506.

Camougrand N, Kissova I, Velours G & Manon S (2004) Uth1p: a yeast mitochondrial protein at the crossroads of stress, degradation and cell death. *FEMS Yeast Res* **5**: 133–140.

Camougrand NM, Mouassite M, Velours GM & Guerin MG (2000) The 'SUN' family: UTH1, an ageing gene, is also involved in the regulation of mitochondria biogenesis in *Saccharomyces cerevisiae*. *Arch Biochem Biophys* **375**: 154–160.

Cox JS & Walter P (1996) A novel mechanism for regulating activity of a transcriptional factor that controls the unfolded protein response. *Cell* **87**: 391–404.

Dubois M, Gilles KA, Hamilton JK, Rebers PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**: 350–356.

Fairn GD, Macdonald K & McMaster CR (2007) A chemogenomic screen in *Saccharomyces cerevisiae* uncovers a

primary role for the mitochondria in farnesol toxicity and its regulation by the Pkc1 pathway. *J Biol Chem* **282**: 4868–4874.

Firon A, Aubert S, Iraqui I, Guadagnini S, Goyard S, Prevost MC, Janbon G & d'Enfert C (2007) The SUN41 and SUN42 genes are essential for cell separation in *Candida albicans*. *Mol Microbiol* **66**: 1256–1275.

Gaigg B, Neergaard TB, Schneiter R *et al.* (2001) Depletion of acyl-coenzyme A-binding protein affects sphingolipid synthesis and causes vesicle accumulation and membrane defects in *Saccharomyces cerevisiae*. *Mol Biol Cell* **12**: 1147–1160.

Gietz RD & Schiestl RH (2007) Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* **2**: 35–37.

Heinisch JJ, Lorberg A, Schmitz HP & Jacoby JJ (1999) The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*. *Mol Microbiol* **32**: 671–680.

Hiller E, Heine S, Brunner H & Rupp S (2007) *Candida albicans* Sun41p, a putative glycosidase, is involved in morphogenesis, cell wall biogenesis, and biofilm formation. *Eukaryot Cell* **6**: 2056–2065.

Hogan DC, Riordan DP, Gerber AP, Herschlag D & Brown PO (2008) Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system. *PLoS Biol* **6**: e255.

Huang D, Moffat J & Andrews B (2002) Dissection of a complex phenotype by functional genomics reveals roles for the yeast cyclin-dependent protein kinase Pho85 in stress adaptation and cell integrity. *Mol Cell Biol* **22**: 5076–5088.

Jansen JM, Wanless AG, Seidel CW & Weiss EL (2009) Cbk1 regulation of the RNA-binding protein Ssd1 integrates cell fate with translational control. *Curr Biol* DOI: 10.1016/j.cub.2009.10.071.

Jo WJ, Loguinov A, Chang M *et al.* (2008) Identification of genes involved in the toxic response of *Saccharomyces cerevisiae* against iron and copper overload by parallel analysis of deletion mutants. *Toxicol Sci* **101**: 140–151.

Kaeberlein M & Guarente L (2002) *Saccharomyces cerevisiae* MPT5 and SSD1 function in parallel pathways to promote cell wall integrity. *Genetics* **160**: 83–95.

Kanki T, Wang K, Cao Y, Baba M & Klionsky DJ (2009) Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Dev Cell* **17**: 98–109.

Kapteyn JC, Ram AF, Groos EM, Kollar R, Montijn RC, Van Den Ende H, Llobell A, Cabib E & Klis FM (1997) Altered extent of cross-linking of beta1,6-glucosylated mannoproteins to chitin in *Saccharomyces cerevisiae* mutants with reduced cell wall beta1,3-glucan content. *J Bacteriol* **179**: 6279–6284.

Kennedy BK, Austriaco NR Jr, Zhang J & Guarente L (1995) Mutation in the silencing gene SIR4 can delay aging in *S. cerevisiae*. *Cell* **80**: 485–496.

Kipnis P, Thomas N, Ovalle R & Lipke PN (2004) The ER-Golgi v-SNARE Bet1p is required for cross-linking alpha-agglutinin to the cell wall in yeast. *Microbiology* **150**: 3219–3228.

Kissova I, Deffieu M, Manon S & Camougrand N (2004) Uth1p is involved in the autophagic degradation of mitochondria. *J Biol Chem* **279**: 39068–39074.

Kissova I, Salin B, Schaeffer J, Bhatia S, Manon S & Camougrand N (2007) Selective and non-selective autophagic degradation of mitochondria in yeast. *Autophagy* **3**: 329–336.

Kitamura K & Yamamoto Y (1972) Purification and properties of an enzyme, zymolyase, which lyses viable yeast cells. *Arch Biochem Biophys* **153**: 403–406.

Lesage G & Bussey H (2006) Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol Mol Biol R* **70**: 317–343.

Lesage G, Shapiro J, Specht CA *et al.* (2005) An interactional network of genes involved in chitin synthesis in *Saccharomyces cerevisiae*. *BMC Genet* **6**: 8.

Mouassite M, Camougrand N, Schwob E, Demaison G, Laclau M & Guerin M (2000) The 'SUN' family: yeast SUN4/SCW3 is involved in cell septation. *Yeast* **16**: 905–919.

Omi K, Sonoda H, Nagata K & Sugita K (2005) Cloning and characterization of psu1(+), a new essential fission yeast gene involved in cell wall synthesis. *Biochem Bioph Res Co* **262**: 368–374.

Okamoto K, Kondo-Okamoto N & Ohsumi Y (2009) Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. *Dev Cell* **17**: 87–97.

Ovalle R, Lim ST, Holder B, Jue CK, Moore CW & Lipke PN (1998) A spheroplast rate assay for determination of cell wall integrity in yeast. *Yeast* **14**: 1159–1166.

Popolo L, Gilardelli D, Bonfante P & Vai M (1997) Increase in chitin as an essential response to defects in assembly of cell wall polymers in the ggp1delta mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **179**: 463–469.

Ram AF, Kapteyn JC, Montijn RC, Caro LH, Douwes JE, Baginsky W, Mazur P, van den Ende H & Klis FM (1998) Loss of the plasma membrane-bound protein Gas1p in *Saccharomyces cerevisiae* results in the release of beta1,3-glucan into the medium and induces a compensation mechanism to ensure cell wall integrity. *J Bacteriol* **180**: 1418–1424.

Roelants FM, Torrance PD & Thorner J (2004) Differential roles of PDK1- and PDK2-phosphorylation sites in the yeast AGC kinases Ypk1, Pkc1, and Sch9. *Microbiology* **150**: 3289–3304.

Roncero C, Valdivieso MH, Ribas JC & Duran A (1988) Isolation and characterization of *Saccharomyces cerevisiae* mutants resistant to Calcofluor white. *J Bacteriol* **170**: 1950–1954.

Saito H & Tatebayashi K (2004) Regulation of the osmoregulatory HOG MAPK cascade in yeast. *J Biochem* **136**: 267–272.

Sosinska GJ, de Groot PW, Teixeira de Mattos MJ, Dekker HL, de Koster CG, Hellingwerf KJ & Klis FM (2008) Hypoxic conditions and iron restriction affect the cell-wall proteome of *Candida albicans* grown under vagina-simulative conditions. *Microbiology* **154**: 510–520.

Valdivieso MH, Ferrario L, Vai M, Duran A & Popolo L (2000) Chitin synthesis in a gas1 mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **182**: 4752–4757.

Velours G, Boucheron C, Manon S & Camougrand N (2002) Dual cell wall/mitochondria localization of the 'SUN' family proteins. *FEMS Microbiol Lett* **207**: 165–172.

Yiannikouris A, Francois J, Poughon L *et al.* (2004) Alkali extraction of β-D-glucans from *Saccharomyces cerevisiae* cell wall and study of their adsorptive properties toward zearalenone. *J Agr Food Chem* **52**: 3666–3673.